DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE) AS A RAPID METHOD FOR ASSESSING GASTROINTESTINAL TRACT MICROFLORA RESPONSES IN LAYING HENS FED SIMILAR ZINC MOLT INDUCTION DIETS

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Accepted for Publication March 1, 2004

ABSTRACT

Induced molting through feed withdrawal can change the microenvironment of crop and ceca sufficiently to allow them to be the sites of Salmonella *colonization in the chicken intestine. This study compares the denaturing gradient gel electrophoresis (DGGE) profiles of microbial crop and cecal communities among molted hens fed similar zinc acetate or zinc propionate amended molt diets to hens either undergoing feed withdrawal or hens full fed and not molted. Dendrograms of DGGE amplicon patterns indicated over 85% similarity of cecal communities between zinc acetate fed hens and zinc propionate fed hens and over 60% similarity of crop communities between zinc acetate fed hens and zinc propionate fed hens. Rapid comparison of complex gastrointestinal microflora profiles in laying hens fed similar diets is possible using DGGE.*

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Journal of Rapid Methods and Automation in Microbiology **12** (2004) 69–81.

INTRODUCTION

Digestive microbial populations in the gastrointestinal tract of adult hens are considered complex but relatively stable (Mead 1989). This complex microbial population is considered resistant to colonization by foodborne pathogens (McNab 1973; Freter 1983a, b). However, hens molted by conventional feed withdrawal may be more susceptible to *Salmonella* enteritidis infection leading to increases in horizontal transfer in flocks (Holt 1992, 1993, 1995; Holt and Porter 1992; Holt *et al.* 1994, 1998). When chickens are undergoing malnutrition or starvation, the pH of crop can increase due to decreased *Lactobacillus* fermentation within the crop (Humphrey *et al.* 1993). Feed withdrawal for 9 days decreases crop lactic acid in conjunction with an increase in crop pH (Durant *et al.* 1999). Feed withdrawal can also lead to decreases in production of acetic, propionic, and total volatile fatty acids (VFA) in the ceca (Corrier *et al.* 1997). These decreased fermentation and production of VFA-producing bacteria present in the ceca colonization may be related to increased susceptibility of molted hens to *S.* enteritidis colonization (Corrier *et al.* 1997). Induced molting through feed withdrawal appears to alter the microenvironment of crop and ceca sufficiently to allow them to be the main sites of *Salmonella* colonization in the chicken intestine (Brownell *et al.* 1970; Soerjadi *et al.* 1981; Impey and Mead 1989).

Previously, we demonstrated that when hens were fed either zinc acetate or zinc propionate amended molting diets *S.* enteritidis colonization was limited in the gastrointestinal tract and fermentation characteristics were similar to full-fed birds (Moore *et al.* 2004). However, some differences in gastrointestinal tract fermentation patterns were observed between trials and among individual birds as well as susceptibility to *S.* enteritidis colonization. Since the two zinc compounds are associated with different organic acids they may elicit different effects on the avian gastrointestinal microflora. Given the importance of the microenvironment on poultry pathogen establishment in the gastrointestinal tract of poultry, it becomes imperative that rapid methods are available to profile the microbial population. Although diet is usually considered a potential key influence on the indigenous gastrointestinal microflora the complexity of the bacterial population and the requirement for anaerobic cultivation techniques makes it difficult to correlate nutritional factors with shifts in specific microbial populations (Ricke and Pillai 1999; Ricke 2003). In addition, there is the potential for overlap in nutritional specificity among groups of gastrointestinal organisms making conventional substrate specific selective media less precise than is desirable for characterizing subtle shifts in microbial populations (Russell and Baldwin 1978, 1979; Russell 1984; Ricke and Pillai 1999).

Conventional DNA-based approaches have been used to provide an imprecise picture of the genetic relatedness of organisms but are less amendable

to more precise characterization (Raskin *et al.* 1997; Ricke and Pillai 1999). More recently Zhu *et al.* (2002) successively used temperature gradient gel electrophoresis to identify 16S rRNA-based gene sequences representing phylogenetic groups in broiler chickens. Hume *et al.* (2003) reported that molecular-based denaturing gradient gel electrophoresis (DGGE) could detect changes in the digestive microbial communities in young chicks and molted laying hens. Our overall objective is to examine detection approaches for potential rapid assessment of microbial profiles from the gastrointestinal tract that could serve as consistent indicators of the presence of a protective microflora against pathogen colonization in molted hens. Therefore, the specific objective of the current study was to compare microbial crop and cecal communities of hens fed either zinc acetate or zinc propionate amended molting diets with hens undergoing feed withdrawal or full fed nonmolted hens using DGGE as described by Hume *et al.* (2003) to assess the reproducibility between diets and independent bird trials.

MATERIAL AND METHODS

Sample Collection

Approximately 0.3 g of cecal contents were collected aseptically in three replicate experiments from five hens each in four treatment groups from a previous study (Moore *et al.* 2004): Group 1 — Control nonmolted hens (C); Group 2 — molted hens with feed removed (Mo); Group 3 — molted hens given a diet containing 10,000 mg of zinc (zinc acetate) per ton of feed (Za); and Group 4 — given a diet containing 10,000 mg of zinc (zinc propionate) per ton of feed (Zp). Volumes were brought to 1 mL with sterile distilled water and samples were stored at -70C until used. Crops from the same hens were collected aseptically and stomached for 30 s in 10 mL of Butterfield's buffer (0.62 mM potassium phosphate, pH 7.2) and 3-mL portions were stored at -70C until used.

Denaturing Gradient Gel Electrophoresis

Methodology for DGGE analysis was conducted as described previously by Hume *et al.* (2003). Briefly, genomic DNA was extracted and isolated (QIAamp DNA Mini Kit, Protocol D; QIAGEN, Valencia, CA) from 1-mL sample volumes of cecal and crop contents. Isolated DNA (50 ng/cecum or crop sample) from each hen was combined to give a total of 250 ng of DNA per group. Primers (50 pmol of each per reaction mixture; primer 2 and primer 3 with a 40-base G-C clamp (Integrated DNA Technologies, Coralville, IA) (Sheffield *et al.* 1989; Muyzer *et al.* 1993) for PCR are shown in Table 1 and

mixed with Jump Start Red-Taq Ready Mix (Sigma Chemical Co., St. Louis, MO), according to methods described in the kit, and 5% (w/v) acetamide to eliminate preferential annealing (Reysenbach *et al.* 1992). Run parameters for amplification (PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, MA) were: (1) denaturation at 94.9C for 2 min; (2) denaturation at 94.0C for 1 min; (3) annealing at 67.0C for 45 s; -0.5C per cycle (touchdown to minimize spurious by-products (Don *et al.* 1991; Wawer and Muyzer 1995); (4) extension at 72.0C for 2 min; (5) repeat steps 2 to 4 for 17 cycles; (6) denaturation at 94C for 1 min; (7) annealing at 58.0C for 45 s; (8) repeat steps 6 to 7 for 12 cycles; (9) extension at 72.0C for 7 min; (10) 4.0C final.

TABLE 1.
OLIGONUCLEOTIDE PRIMER SEQUENCES

Primer Designation[1]	Primer Sequences (5'→3'')
Primer 2	ATTACCGCGGCTGCTGG
Primer 3	GCCCGCCGCGCGCGGCGGGCGGGGCGGGGG-CACGGGGGGCCTACGGGAGGCAGCAG

[1] Based on published sequences (Sheffield *et al.* 1989; Muyzer *et al.* 1993) and primers were obtained from Integrated DNA Technologies, Inc. (Coralville, IA).

Amplicons were separated on polyacrylamide gels (8% (vol/vol) acrylamide-bisacrylamide ratio 37.5:1 (Bio-Rad Laboratories; Richmond, CA) cast with a 35 to 60% urea-deionized formamide (Sigma) gradient; 100% denaturing acrylamide was 7 M urea and 40% deionized formamide. Samples were mixed with an equal volume of 2X loading buffer (0.05% (wt/vol) bromophenol blue, 0.05% (wt/vol) xylene cyanol, and 70% (vol/vol) glycerol) and 4 mL of each was loaded in sample wells. Gel electrophoresis was run in a DCode Universal Mutation Detection System (Bio-Rad) with 0.5X TAE (20 mM Tris (pH 7.4), 10 mM sodium acetate, 0.5 M EDTA) run buffer at 59C for 17 h at 60 V. Bands were stained with SYBR Green I (Sigma) (1:10,000 dilution) and fragment pattern relatedness was determined with Molecular Analysis Fingerprinting Software, version 1.6 (Bio-Rad Laboratories, Hercules, CA) based on the Dice similarity coefficient and the unweighted pair group method using arithmetic averages for clustering.

RESULTS AND DISCUSSION

Given the problems associated with conventional feed withdrawal induced molting and *S.* enteritidis colonization, there is a need to apply molecular characterization as a rapid detection tool for screening the effectiveness of alternative molting diets to select indigenous gastrointestinal microflora that consistently limit *S.* enteritidis colonization and invasion. Zinc propionate (10,000 ppm zinc) as an alternative molting diet additive has been recently demonstrated to induce molt (Moore *et al.* 2004; Park *et al.* 2004) but Zp fed hens were more susceptible to *S.* enteritidis colonization compared to Za fed hens (Moore *et al.* 2004). Since these two compounds are both zinc-based organic acids the question arises as to whether detectable differences are present in the gastrointestinal microbial populations supported by these respective diets that could account for the differences in *S.* enteritidis colonization. Therefore, DGGE profiles were generated for the 2 key colonization sites for *S.* enteritidis, namely the crop and cecum. An important criteria for determining the gastrointestinal microbial population that may be an indicator of microflora selected by consumption of a particular diet is the consistency of the molecular patterns in independent trials. In the present study, laying hen trial crop and cecal samples were collected from 3 independent laying hen molting trials conducted in a previous study (Moore *et al.* 2004) to compare the microbial populations in the crops and ceca in birds undergoing molt induction via Za or Zp amended diets versus the more extreme dietary manipulation of either complete feed withdrawal or hens continued to be fed laying ration *ad libitum.*

The ceca is an alimentary tract site in poultry that is most likely to be colonized by *Salmonella* (Fanelli *et al.* 1971) and *S.* enteritidis replicates and disseminates to various organs, including the ovaries (Gast and Beard 1990; Shivaprasad *et al.* 1990). An increase on the level of cecal colonization *Salmonella* can result from feed withdrawal (Moran and Bilgili 1990; Ramirez *et al.* 1997). The native intestinal microflora are believed to play an important role in preventing *Salmonella* colonization of the cecum (Nurmi and Rantala 1973; Barnes *et al.* 1980; Nisbet *et al.* 1994; Corrier *et al.* 1995) in the chicken. The importance of VFA and pH in preventing *Salmonella* colonization of the cecum has been associated with increased VFA concentrations and decreased pH (Barnes *et al.* 1979; Nisbet *et al.* 1994; Corrier *et al.* 1995). However, Corrier *et al.* (1997) reported that induced molting by feed withdrawal had no apparent effect on pH or on the oxidation-reduction potential of ceca. This would indicate that changes in the cecal microflora are somewhat subtle in response to dietary changes and potentially difficult to detect metabolically.

Based on DGGE analysis, cecal populations changed as a result of feed removal (Mo) and by the inclusion of feed. Cecal populations from hens given Za and Zp shared the greatest similarities as indicated by coefficients of 86.9

and 85.3%, respectively, for trials 1 and 2 but in trial 3, cecal populations from hens undergoing Mo or fed Zp shared the greatest similarity (90.1%). Microbial patterns from Za, Zp, and Mo hens formed a linked group for trials 1 and 3, but C, Za, and Zp hens formed a more related group for trial 2. Recovery of *S.* enteritidis from these ceca samples in our previous study (Moore *et al.* 2004) did not reveal consistent differences in the number of bird ceca positive for *S.* enteritidis but somewhat higher *S.* enteritidis log counts were recovered from trial 2 birds and in some cases trial 3 birds. When these ceca samples were examined for fermentation products from the same trials and reported in the previous study (Moore *et al.* 2004) there were negligible differences in most cecal individual VFA, total VFA, and lactic acid concentrations among Za or Zp molted hens in all 3 trials. However, in trial 3, hens undergoing feed withdrawal exhibited similar lower levels of butyric acid concentrations as the Zp fed hens versus Za fed hens (Moore *et al.* 2004). The DGGE relatedness of cecal populations among nonmolted hens, molted by feed withdrawal, and molted by Za or Zp determined in this study for the most part appeared to match the similarities in fermentation profiles observed previously for these same trials (Moore *et al.* 2004).

The crop can be one of the main reservoirs for *Salmonella* (Hargis *et al.* 1995), and feed withdrawal can increase the number of chickens with crops colonized by *Salmonella* (Ramirez *et al.* 1997). Humphrey *et al.* (1993) reported that an increase in the recovery of *S.* enteritidis from the crop of broilers resulted from feed deprivation for 24 h. Durant *et al.* (1999) reported that the introduction of *S.* enteritidis into the crop environment with high pH and lowered concentrations of lactate and total VFA were accompanied by increased crop colonization. In the current study, DGGE analysis revealed the highest similarity in crop microbial populations (82.8, 79.1, and 73.2%) in C vs Za hens (trial 1), Za vs Zp hens (trial 2), and C vs Zp hens (trial 3), respectively. In all 3 trials, greater similarity in crop DGGE profiles was shared between control hens and hens fed dietary zinc than with profiles of crops from feed withdrawal molted hens. Recovery of *S.* enteritidis from these crop samples in our previous study (Moore *et al.* 2004) did reveal consistently low numbers of bird crops positive for *S.* enteritidis in trial 3 compared to trials 1 and 2 and in some cases higher *S.* enteritidis colony forming units were recovered from trial 1 and 2 birds. When the crop samples were examined for pH and fermentation products from the same trials and reported (Moore *et al.* 2004) there were negligible differences in crop pH and lactic acid production between hens fed Za and hens fed Zp in all 3 trials while crop pH levels were similar only in trials 1 and 2. Crop lactic acid was generally less for Mo hens than C hens or Za and Zp molted hens (Moore *et al.* 2004). Consequently, the general similarity of crop microbial populations for hens receiving feed may in part be due to comparable fermentative crop microflora yielding similar lactic acid production

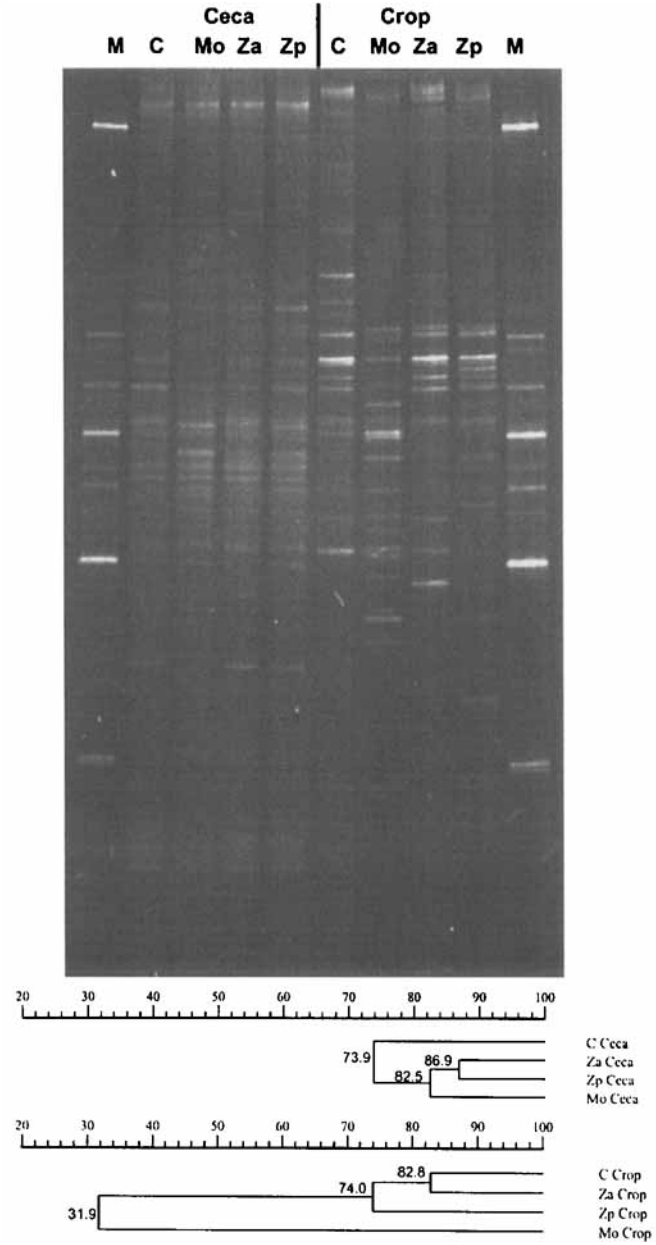

FIG. 1. DENATURING GRADIENT GEL ELECTROPHORESIS OF CECA OR CROP BACTERIAL 16S AMPLICON PATTERNS FROM LEGHORN HENS ON NONMOLTED CONTROL (C), MOLTED FEED WITHDRAWAL (Mo), ZINC ACETATE (Za), AND ZINC PROPIONATE (Zp) IN TRIAL 1

M refers reference amplicons. Relative similarity of band patterns is indicated by their grouping on the dendrogram and the percentage coefficient.

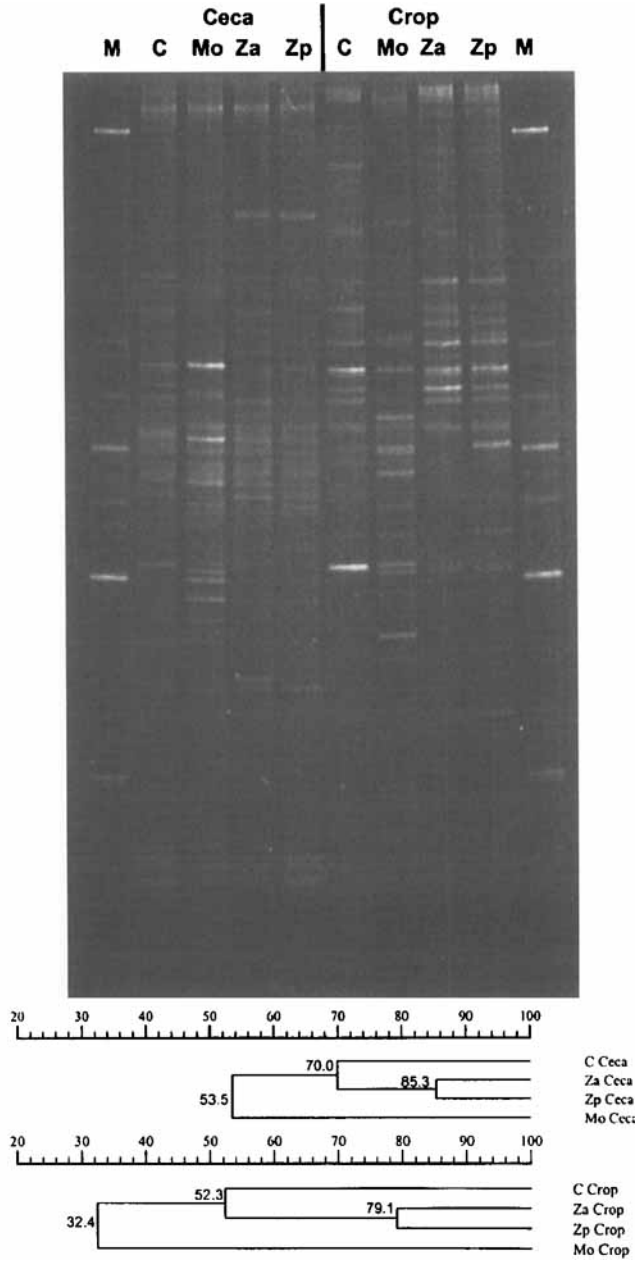

FIG. 2. DENATURING GRADIENT GEL ELECTROPHORESIS OF CECA OR CROP BACTERIAL 16S AMPLICON PATTERNS FROM LEGHORN HENS ON NONMOLTED CONTROL (C), MOLTED FEED WITHDRAWAL (Mo), ZINC ACETATE (Za), AND ZINC PROPIONATE (Zp) IN TRIAL 2

M refers reference amplicons. Relative similarity of band patterns is indicated by their grouping on the dendrogram and the percentage coefficient.

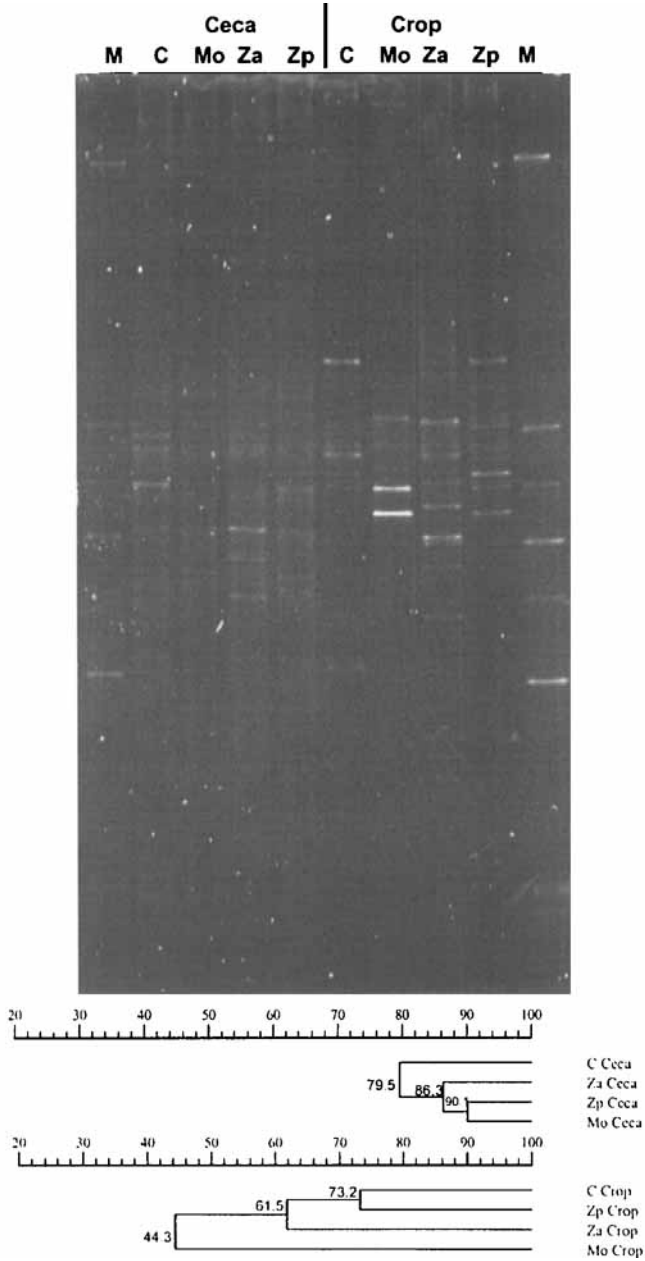

FIG. 3. DENATURING GRADIENT GEL ELECTROPHORESIS OF CECA OR CROP BACTERIAL 16S AMPLICON PATTERNS FROM LEGHORN HENS ON NONMOLTED CONTROL (C), MOLTED FEED WITHDRAWAL (Mo), ZINC ACETATE (Za), AND ZINC PROPIONATE (Zp) IN TRIAL 3

M refers reference amplicons. Relative similarity of band patterns is indicated by their grouping on the dendrogram and the percentage coefficient.

and leading to similar pH levels. The DGGE detected difference in feed withdrawal hens may result from lack of feed possibly leading to a decrease in the *Lactobacilli* population in hens that are deprived of feed (Durant *et al.* 1999; Humphrey *et al.* 1993).

Based on the results of the current study, molecular-based denaturing gradient gel electrophoresis (DGGE) method can be applied as a rapid screening tool to detecting similarities in the digestive microbial communities between molted hens by either Za or Zp amended feeds. However, greater molecular sensitivity may be needed to more precisely quantitate key indicator groups of gastrointestinal bacteria that reveal the potentially more subtle differences created by feeding similar molting diets.

ACKNOWLEDGMENTS

This research was supported by Kemin Americas, Inc., Des Moines, IA, TAES project G-8815, Hatch grant H8311 administered by the Texas Agricultural Experiment Station and USDA-NRI grant number 2001-02614. S. Y. Park was supported by a Pilgrim's Pride (Pittsburg, TX) endowed graduate fellowship. The authors thank Charles Hernandez and Clayton Myers (USDA-ARS, College Station, TX) for technical support.

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